Antiviral Therapy **8**:373-377

Protection from HIV-1 infection of primary CD4 T cells by CCR5 silencing is effective for the full spectrum of CCR5 expression

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Stable gene silencing by RNA interference (RNAi) can be achieved by expression of small hairpin RNAs (shRNAs) from RNA polymerase III promoters. We have tested lentiviral vectors expressing shRNAs targetting CCR5 in primary CD4 T cells from donors representing various CCR5 and CCR2 genetic backgrounds covering the full spectrum of CCR5 expression levels and permissiveness for HIV-1 infection. A linear decrease in CCR5 expression resulted in a logarithmic decrease in cellular infection, giving up to three logs protection from HIV-1 infection *in vitro*. Protection was maintained at very high multiplicity of infection. This and other recent reports on RNAi should open a debate about the use of RNAi gene therapy for HIV infection.

Introduction

RNA interference (RNAi) is a conserved biological process that results in degradation of the targeted mRNA [1]. It can be harnessed to silence (knock-down) genes selectively by expressing a shRNA containing a sequence matching the targeted mRNA [2]. The hairpin RNA is processed to 21 nt long molecules (siRNAs) that are loaded onto the RNA-induced silencing complex (RISC), which then cleaves the target mRNA. RNAi has previously been used to degrade HIV-1 transcripts [3–8], but it also provides the opportunity to assess the role of host genes in HIV-1 infection and provides new avenues for therapeutic intervention. Knock-down of cellular proteins needed for HIV-1 infection or replication, including CD4, CCR5, CXCR4, NF-κB p65 and Tsg101, has been achieved recently by transfection of siRNA oligonucleotides or plasmids expressing shRNAs [4,9–12]. A limitation of the technique has been the dependence on transfection for delivery of oligonucleotides or plasmids. Only certain cell lines can be transfected, and efficient transfection into primary cells is virtually impossible. In contrast, retroviral delivery of siRNA is effective in most cell lines and many primary cells [13,15].

We have used lentiviral delivery of shRNA targeted against CCR5 to prevent HIV infection of primary CD4 T cells from donors representing several CCR5 genetic backgrounds and covering the full spectrum of CCR5 expression. We define the level of CCR5 required for productive infection of primary cells and show that there is a logarithmic response to decrease in receptor density. This and previous studies [3–9,11,12,15] represent the first steps in the development of RNAi therapy for HIV infection.

Methods

Donor cells, CCR5 expression

Human CD4 T cells (Table 1) were isolated by anti-CD4 magnetic beads (Miltenyi Biotech, Germany) from 12 healthy blood donors and expanded *in vitro* by using phytohaemagglutinin (PHA), interleukin-2 (IL-2) and irradiated PBMCs. They were cultured in RPMI (GIBCO-Invitrogen, Basel, Switzerland) with 20% fetal calf serum (FCS) and 20 U/ml of human IL-2, at 37°C and 5% CO_2. Characterization included determination of *CCR5* (Δ32 deletion and promoter p1/59029A alleles) and *CCR2* (64I allele) genotype, permissiveness to HIV-1 infection (see below), and flow cytometry (FACS) of CCR5 expression by using a phycoerythrin-conjugated anti-CCR5 monoclonal (BD Bioscience, Basel, Switzerland). FACS results were expressed as relative mean fluorescence intensity (MFI, ratio CCR5/isotype mean intensities) and as percentage of CCR5-positive cells.

Design of the siRNAs

Hairpin siRNA expression cassettes were constructed by annealing two complementary 64 bp oligonucleotides with the following generic structure: 5′-GATCCCCsense-targetTTCAAGAGAantisense-

Table 1. Baseline characteristics of donor CD4 T cells

	Genotype				
Donor	*CCR5*	*CCR5* promoter	*CCR2*	CCR5 expression (% positive cells)	Permissiveness (p24 pg/ml)*
61	Δ32/Δ32	p1/p1	wt/wt	0	66
18	Δ32/Δ32	p1/p1	wt/wt	0	1
11	wt/Δ32	p1/p1	wt/64I	0	1572
70	wt/wt	p1/p1	wt/wt	3.8	289000
13	wt/wt	wt/wt	wt/wt	6.6	13082
110	wt/wt	wt/p1	wt/wt	7.1	14871
106	wt/wt	wt/p1	wt/64I	11.3	17891
94	wt/wt	wt/wt	wt/wt	14.2	171990
101	wt/wt	wt/p1	wt/64I	14.2	10752
42	wt/wt	p1/p1	wt/64I	18.9	194800
81	wt/wt	wt/wt	wt/wt	29.8	158310
22	wt/wt	wt/wt	wt/wt	40.0	167740

wt, wild-type; Δ32, deletion in CCR5; p1, 59029A allele in *CCR5* promoter; 64I, isoleucine in CCR2 codon 64.
*7-Day data, non-expanded cells, average from duplicate experiments.

targetTTTTTGGAAA-3′ and 5′-AGCTTTTCCAA AAAsense-targetTCTCTTGAAantisense-targetGGG-3′. The sense target sequence has a guanine or a cytosine at position 1, as recommended for efficient transcription initiation from the H1 promoter [16]. The hairpin has been designed to be an optimal substrate for Dicer, including flanking the target sequence by a 5′ AA, and a 3′ TT, a greater than 30% GC content, a TTCAAGAGA loop and a TTTTT terminator recognized by RNA polymerase III [17]. Annealing of forward and reverse oligonucleotides reconstitute the restriction sites *Bgl*II and *Hind*III. CCR5 sense targets are: LV466 (5′-GTGTCAAGTC-CAATCTATG), LV468 (5′-TCAATGTGAAGCAAA TCGC), LV470 (5′-GAGCATGACTGACATCTAC), LV472 (5′-GGTCTTCATTACACCTGCA), LV474 (5′-CATTGTCCTTCTCCTGAAC). The oligonucleotides were cloned into the *Bgl*II and *Hind*III sites in pSUPER [17].

Lentivirus production and transduction

A *Bam*HI–*Sal*I restriction fragment carrying the H1 promoter and the shRNA expression cassette from the pSUPER plasmids was ligated into the lentiviral vector pAB286, which has a puromycin selection marker [14]. All constructs were confirmed by restriction enzyme digestion and sequencing. The packaging cell line 293T was transfected with four plasmids: pAB286 containing each shRNA, pMDLg/pRRE encoding HIV-1 Gag and Gag/Pol, pRSVrev encoding HIV-1 Rev and the pMD.G encoding VSV-G envelope protein (kind gift from D Trono, www.tronolab.unige.ch). A lentivirus carrying the green fluorescent protein (LVeGFP) was included in the experiments to assess efficiency of transduction.

Analysis of kinetics of CCR5 silencing was done by using Ghost-CCR5 cells (Central facility for AIDS Reagents, NIRSC, MRC, UK). Transduction was performed by spinoculation of 30000 cells in 300 µl culture medium with 150 µl of lentivirus-containing supernatant in the presence of 20 µg/ml polybrene (Sigma, Buchs, Switzerland) for 3 h at 1500 g at 22°C. Donor CD4 T cells were stimulated for 2 days with PHA (2 µg/1 ml) and IL-2 (20 U/ml), and 600 000 cells were transduced with the various lentiviral constructs in the presence of 2 µg/ml polybrene and spinoculated as above. After 48 h, transduced cells were selected overnight with 2 µg/ml puromycin, followed by 3 days at 1 µg/ml. Changes in GHOST-CCR5 or primary CD4 T cells CCR5 surface expression was monitored by FACS. 10000 events were analysed using FACScalibur with the CellQuest software (Becton Dickinson, Basel, Switzerland).

HIV-1 infection

CD4 T cells (1.5×10^5 cells) were infected with the R5 strain NL4-3Ba*Lenv* (500 pg p24 antigen) 6 days after transduction. After 5, 7 and 12 days of infection, p24 was measured in the supernatant. Analysis of the degree of protection conferred by CCR5 silencing included exposure of primary CD4 T cells to 1 and 2 log higher inocula; 5000 and 50000 pg p24 antigen per 1.5×10^5 cells, respectively.

Results

Kinetics of RNAi of CCR5

The efficiency of RNAi is known to depend on the exact choice of sequence within the mRNA targeted. The efficiency of the different siRNA lentiviruses was evaluated by flow cytometry for CCR5 in GHOST-CCR5 cells.

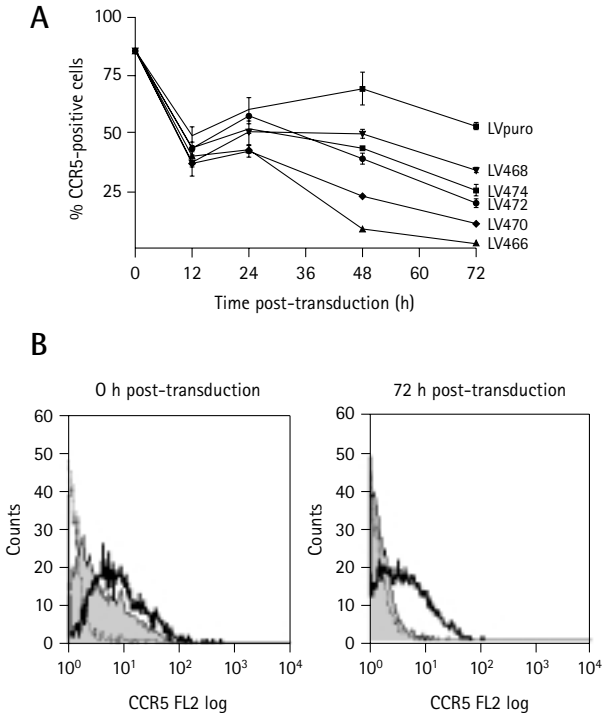
The transduction efficacy determined with a GFP-expressing lentivirus (LVeGFP) was 95%. After a transient non-specific reduction in CCR5 expression associated with spinoculation, the different siRNA lentiviruses resulted in CCR5 silencing ranging, at 72 h post-transduction, from 28% (LV468) to 88% (LV466) when compared with control LVpuro-transduced cells (Figure 1).

Silencing CCR5 in primary cells

The most effective siRNA lentivirus, LV466, was used to transduce a bank of purified CD4 T cells from healthy blood donors. With the exception of donor cells #94 and #81, the susceptibility to infection of the expanded pools of banked cells was similar to that of non-expanded cells, and prolonged IL-2 stimulation modified to some extent baseline CCR5 expression (data not shown). The transduction efficacy determined with LVeGFP was 25–50%. Non-transduced cells were killed by selection for the puromycin resistance gene on the lentivirus. At this level of infection we expect each T cell to contain only a single copy of the shRNA vector. LV466 diminished CCR5 surface expression (mean fluorescence intensity, MFI) by 55 to 90%, and decreased the number of CCR5-positive cells to less than 1%, with the exception of donor cells #22, which retained 2% of residual positive cells (Figure 2A and B). Silencing resulted in high degree protection from infection (Figure 2C). Overall, LV466 was able to diminish HIV-1 infection of primary cells by up to 3 logs. The amount of p24 present in the supernatant of donor cells #13, #101, #42, #110, #11, #94 was 84–785 pg/ml. This was greater than that observed for the two homozygous Δ32 donor cells (48 and 92 pg/ml, corresponding to the input inoculum). The p24 level stabilized or decreased between days 5, 7 and 12 post-infection (range 53–755 pg/ml), suggesting a high level protection or aborted infection. Despite maintaining a significant degree of protection, donors #70 and #22 displayed an increase in viral replication over time (Figure 2C).

Protection conferred by CCR5 silencing was independent from HIV-1 inoculum size. Multiplicity of infection ranging from MOI 0.007 to 7 of different donors cell pools (#13, #42 and control Δ32 homozygous #18) did not result in a replication breakthrough.

Figure 1. Kinetics of CCR5 silencing post-transduction of shRNA lentiviruses into GHOST-CCR5 cells



(A) LV466-LV474 express siRNA against various CCR5 mRNA targets, resulting in various levels of silencing efficacy; **(B)** flow cytometry histogram of CCR5 expression at 0 and 72 h post-transduction, illustrating the specific reduction in surface CCR5 in cells transduced with LV466 (filled grey) as compared to LVpuro (black, unfilled). The isotype is indicated by a thin broken line. LVpuro is the control vector with no shRNA insert.

Relationship between CCR5 expression and infection

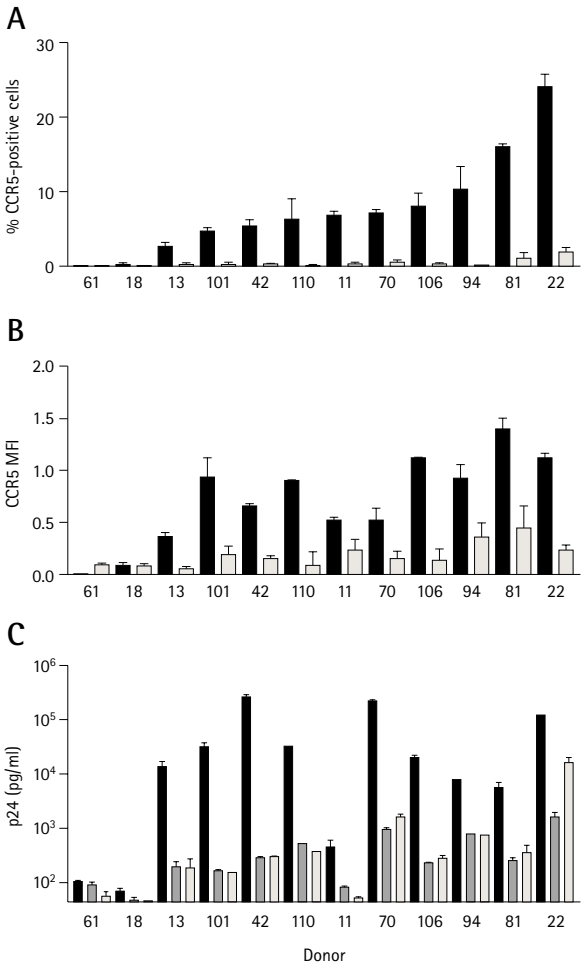
The availability of lentiviral constructs that exhibited intermediate efficacy in silencing CCR5 allowed investigation of the relationship between CCR5 surface density and susceptibility of CD4 T cells to infection. For this purpose, LV466 (88% silencing in GHOST-CCR5), LV470 (67%), LV472 (44%) and LV474 (37%) were used to infect CD4 T cells from four different donors (two permissive in the 10^5 p24/ml range and two in the 10^4 p24/ml level). A linear decrease in CCR5 density was associated with a logarithmic decrease in cell infection. In the experimental context of RNAi and IL-2 stimulation, a putative threshold for productive infection was observed at 1% of CCR5 cells (Figure 3).

Discussion

This, and the recent report by Qin *et al.* [15], demonstrate that stable RNAi can be effectively achieved in primary CD4 T cells, a key target of HIV-1. Overall, CCR5 silencing was effective for a collection of primary CD4 T cells representing a wide spectrum of genotypes, CCR5 surface expression and degree of permissiveness to HIV-1 infection. However, cells with the highest expression of CCR5 showed escape from suppression of viral infection with time, thus underscoring the need to further improve delivery, integration or expression of lentiviral vectors.

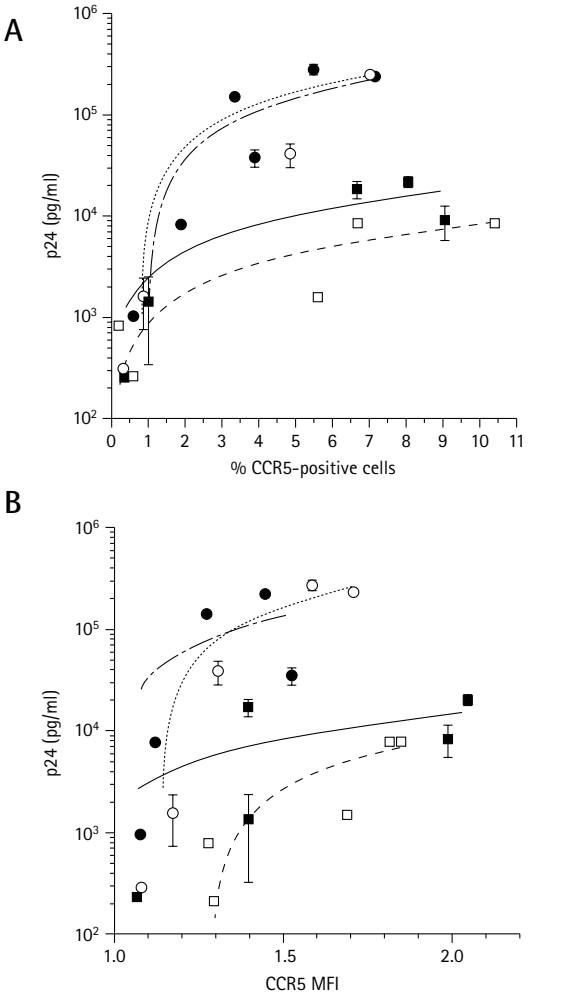
We were intrigued by the observation that complete inhibition of CCR5 surface expression was not needed to limit productive infection. Protection was observed

Figure 2. CCR5 silencing and protection from HIV-1 infection of primary CD4 T cells



The most effective lentivirus, LV466, was used to transduce a collection of CD4 T cells representative of the various *CCR5/CCR2* genotypes, and the spectrum of CCR5 surface expression and permissiveness to infection. Silencing of CCR5 was measured by flow cytometry analysis after transduction with control vector LVpuro (black bars) or LV466 (grey bars), and expressed as percentage of CCR5-positive cells (**A**), and relative mean fluorescence intensity (**B**). (**C**) HIV-1 susceptibility of CD4 T cells transduced with LVpuro at day 7 post-infection (black bars) or LV466 at day 7 (dark grey), and day 12 post HIV-1 infection (light grey). Shown are mean ±SEM values.

Figure 3. Logarithmic correlation between silencing efficacy and protection from infection



CD4 T cells from four donors (#42, #70, #94, #106) were transduced with different lentiviruses representing the spectrum of efficacy of CCR5 silencing or with control vector LVpuro. For a same donor pool, results from the separate transduction/infection experiments are plotted together. Shown are individual values and the regression curve for each individual donor (#42, open circles + point line; #70, black circles + point-dashed line; #94, open squares + dashed line; #106, black squares + full line). Results are shown as percentage of CCR5-positive cells (**A**) or as surface CCR5 expression (**B**), as represented by the relative mean fluorescent intensity (MFI). p24 was assessed at 7 days post-infection.

despite the presence of cells expressing CCR5 at the surface. This suggests that other factors may limit viral entry, such as the level of endogenous β-chemokines in the *in vitro* system, that infection is aborted post-entry, or that predator–prey dynamics allow uninfected cells to overgrow the culture. Venkatesan *et al.* [18] pointed out that the minimal threshold CCR5 level for productive HIV infection of PBMCs is largely unknown, since *in vivo* levels of CCR5 on circulating PBMCs may be modulated by many unidentified factors relating to immune challenge and inflammation. To investigate this issue, we used lentiviral–siRNA constructs that led to various degrees of CCR5 silencing. By transducing the same donor CD4 T cell pools with different constructs it was possible to knockdown CCR5 in a stepwise manner in the same genetic background. A linear decrease in CCR5 expression led to a logarithmic decrease in susceptibility to infection. Use of quantitative (Q)FACS would help define the minimum level of CCR5 needed for productive infection [19].

The high efficacy of CCR5 and viral gene silencing [3–8,11,15] raises the possibility to use gene therapy to treat HIV infection. The tools for stable transduction of siRNA into primary cells, stem cells or specific tissues are available [20], as is an accumulating experience of gene therapy in humans. The risk of insertional mutagenesis precludes the use of retroviral vectors in healthy or asymptomatic HIV-infected individuals [21].

However, proof-of-concept trials aimed at transducing peripheral cells from patients who have failed all existing antiretroviral therapies should be considered.

Acknowledgements

We thank D Trono for useful comments, G Bleiber, R Martinez and C Suarez for support, and P Romero for help in setting the procedure for cell expansion. Funded by the Leenaards Foundation and by the Swiss National Science Foundation.

Christophe Butticaz and Angela Ciuffi contributed equally to this study.

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Received 23 April 2003; accepted 12 May 2003